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Factors Promoting 'In vitro' Excystation of 'Giardia muris' Cysts

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16. ABSTRACT

Giardia muris cysts, isolated from mouse feces, excysted routinely at levels greater than 90%, when induced in 1X Hanks' supplemented with 17 mM glutathione, 29 mM L-cysteine-HCl, and 50 mM $NaHCO_3$ for 30 minutes at 35°C, followed by washing and suspension in trypsin-Tyrode's solution at pH 8.0. Although trypsin was not required in this final step, it enhanced the escape of the trophozoites from their cysts. *Giardia muris* excystation was dependent upon the length of the induction period, pH, oxidation-reduction potential, and temperature. Optimal induction conditions for excystation were: an induction period of 5 to 30 minutes; pH of 2; 120 mV oxidation-reduction potential; and a temperature around 35°C. A gradual decline in excystation occurred as pH and oxidation-reduction potential were changed to 7 and 57 mV, respectively. There was a pronounced increase in excystation percentages with increasing temperatures between 0 and 37°C. At 40°C and above, the *G. muris* cysts showed signs of inactivation. The thermal death point of *G. muris* cysts was determined to be about 54°C. *Giardia muris* cysts showed no polarity; however, the tail or posterior trophozoite portion always emerged through one end of the cyst first. Cytokinesis began within the first hour post excystation. This method always produced extremely active, normal looking *G. muris* trophozoites.

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Factors promoting *in vitro* excystation of *Giardia muris* cysts*

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Summary

Giardia muris cysts, isolated from mouse faeces, excysted routinely at levels greater than 90%, when induced in 1X Hanks' supplemented with 17 mM glutathione, 29 mM L-cysteine-HCl, and 50 mM $NaHCO_3$ for 30 minutes at 35°C, followed by washing and suspension in trypsin-Tyrode's solution at pH 8·0. Although trypsin was not required in this final step, it enhanced the escape of the trophozoites from their cysts. *G. muris* excystation was dependent upon the length of the induction period, pH, oxidation-reduction potential and temperature. Optimal induction conditions for excystation were: an induction period of 5 to 30 min; pH of 2; 120 mV oxidation-reduction potential; and a temperature around 35°C. A gradual decline in excystation occurred as pH and oxidation-reduction potential were changed to 7 and 57 mV, respectively. There was a pronounced increase in excystation percentages with increasing temperatures between 0 and 37°C. At 40°C and above, the *G. muris* cysts showed signs of inactivation. The thermal death point of *G. muris* cysts was determined to be about 54°C. *G. muris* cysts showed no polarity; however, the tail or posterior trophozoite portion always emerged through one end of the cyst first. Cytokinensis began within the first hour after excystation. This method always produced extremely active, normal-looking *G. muris* trophozoites.

Introduction

In the past 15 years numerous waterborne giardiasis outbreaks, affecting thousands of people, have been reported in the USA (LIPPY, 1981; CRAUN, 1979). The areas in which these outbreaks have occurred have been served by surface drinking water which has received minimal treatment. Generally, these surface waters have been free of gross human faecal contamination and subjected to chlorination. Beavers have been epidemiologically implicated in some outbreaks, and cross-species transmission from beavers to man has been indicated by DAVIES & HIBLER (1979).

After detecting *Giardia* cysts in drinking water, determining if they are infective and how much disinfectant is required to kill them are of interest. Initially we approached these problems using cysts from human donors but were forced to abandon this approach due to our inability to obtain consistent supplies of cysts. As a substitute for *G. lamblia* cysts we have used *G. muris* cysts from laboratory mice (ROBERTS-THOMSON *et al.*, 1976).

The question of cyst viability may be addressed by induction of excystation. Excystation of *G. lamblia* cysts has been described by several investigators. The pioneering work was reported by HEGNER (1925, 1927a, b, c) and DESCHIENS (1929). BINGHAM & MEYER (1979) and BINGHAM *et al.* (1979) have elucidated a more defined procedure for *in vitro* induction of *G. lamblia* excystation, but we were unable to excyst *G. muris* cysts using their procedure. The present study reports a procedure for optimal *in vitro* excystation of *G. muris* cysts which has been reported in abstract form earlier (SCHAEFER *et al.*, 1980).

Materials and Methods

G. muris cysts were obtained from female Swiss albino mice (CF-1) using the cycle maintenance and cyst purification procedures developed by ROBERTS-THOMSON *et al.* (1976). Weekly, each of 20 mice were infected *per os* with 1000 cysts suspended in 0·2 ml distilled water. The specific pathogen-free mice were obtained from Charles River Breeding Laboratories, Inc. in Portage, Michigan, USA. The *G. muris* strain was obtained from Drs. Erik Hewlett and John Andrews at Case Western Reserve School of Medicine. This strain was originally isolated from a naturally infected golden hamster, and has been maintained in our laboratory for three years. Purified cysts were stored in distilled water at 4°C and were used within five days of isolation unless otherwise stated. Cyst suspension densities were determined with an American Optical phase haemocytometer and usually were adjusted to $1·0 \times 10^6$ cysts/ml.

Various combinations of induction, wash and incubation solutions were utilized in an attempt to define the components that yielded the maximal excystation percentage. The induction solutions consisted of: (i) 0·01 M aqueous hydrochloric acid (HCl); (ii) pepsin digest solution (PEP-NaCl) made according to the formulation of VON BRAND *et al.* (1952); (iii) reducing solution (MEYER, 1976) plus 0·1 M sodium bicarbonate (RS). The wash and incubation solutions were as follows: (i) HSP-3, pH 7·0 (MEYER, 1976); (ii) HSP-3 plus mouse bile prepared by placing the contents of two to four CF-1 female mice gall-bladders in a graduated tube and bringing the volume to 12 ml with HSP-3 (HSP-3B); (iii) 0·5% (W/V) trypticase and 0·5% (W/V) glucose dissolved in distilled water (TRYP-GLU); and (iv) 0·5% (W/V) trypsin dissolved in 1X Tryode's solution (PARKER, 1950) (TRY-TYR).

The trypsin-Tyrode's solution is difficult to make, because the lyophilized trypsin (1:100, hog pancreas, ICN Parmaceuticals, Inc.) does not completely dissolve. The trypsin was vigorously mixed on a magnetic stir plate for 30 min. Undissolved trypsin was pelleted by centrifugation at 21,000 *g* for 10 min. The supernatant was filtered through a 0·45 μm Millipore filter by positive pressure. Unless otherwise stated, the pH of the trypsin-Tyrode's solution was adjusted to 8·0. In one instance the trypsin-Tyrode's solution was heated in a 100°C water bath for 10 min to inactivate the trypsin (TRY-TYR_h). Either 1·0 N or 0·1 N aqueous hydrochloric acid or 7·5% (W/V) sodium bicarbon-

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ate were used for pH adjustments for the various solutions. The pH change was followed on a Beckman model SS-2 pH meter. Oxidation-reduction potentials were measured using a Corning model 125 meter equipped with a Brinkmann metrohm U/3M Ag/AgCl electrode.

The BINGHAM-MEYER (1979) procedure for excysting *G. lamblia* cysts can be subdivided into several steps: induction, wash and incubation. It was on the basis of these three steps that the *G. muris* excystation was developed. Induction was done in 15 ml screw cap tubes containing 0·5 ml cyst suspension and 10 ml induction medium with incubation in a 37°C water bath for 30 min. Optimal induction was obtained using 5 ml 1X Hanks' supplemented with 17 mM glutathione, 29 mM L-cysteine-HCl (reducing solution) and 5 ml 50 mM $NaHCO_3$. It should be stated that the reducing solution pH was 2 and was never adjusted. Following induction, the *G. muris* cysts were pelleted by centrifugation in a swinging bucket rotor at ambient temperature for 2 min at 300 *g*. After aspiration of the supernatant, the wash step was begun by resuspension of the cysts by vortexing in 10 ml of the wash solution. To begin the incubation stage, the cysts were removed from the wash solution by centrifugation as above and were resuspended in 0·5 ml of the incubation solution. Incubation was done in depression slides sealed with coverslips and Vaspar as described by BINGHAM *et al.* (1979) for 30 min in a warm air incubator at 37°C. Optimal wash and incubation steps were obtained using trypsin-Tyrode's solution, pH 8. The inverted depression slides were scanned on a Zeiss inverted microscope to determine the percentage excystation. Unlike the Bingham-Meyer procedure, full and empty cysts were counted instead of excysted trophozoites and full cysts. Calculation of the percentage excystation was done using a differential cell counter and the following equation:

$$\text{percentage excystation} = \frac{\text{ECW} + \text{PET}}{\text{ECW} + \text{PET} + \text{IC}} \times 100,$$

where ECW is the number of empty cyst walls, PET is the number of partially excysted trophozoites and IC is the number of intact cysts. Each percentage excystation determination involved a total of 100 cyst types.

Results

Typical results showing the effect of varying induction, wash and incubation solutions on *G. muris* excystation are shown in Fig. 1. The variations were attempts to simulate mouse gastrointestinal tract conditions. Underneath each bar on the abscissa are three lines of description. In descending order, these lines represent the solution used in the induction step, wash step and incubation step. pH of all induction solutions was approximately 2. Using the Bingham-Meyer procedure (bar A) only 2 to 3% of the *G. muris* cysts ever excysted. When the induction step was changed to a pepsin digest solution, excystation increased to about 25% (bar B). Bar C shows an excystation increase to about 40% with mouse bile included in the incubation step. In these first three cases, the pH of the HSP-3 was between 6·9 and 7·1. When a trypsin-Tyrode's solution was used for the wash and incubation steps, the result was quite pronounced and is shown in bar D. Excystation increased to over 90%, when reducing solution and 0·1 M sodium bicarbonate (1:1) were used in place of the pepsin digest (bar E). The mixing of the reducing solution and bicarbonate always resulted in CO_2 evolution. Heat inactivation of the trypsin depressed excystation to 78% (bar F). Proteinaceous material at pH 7·5 did not substitute for the trypsin solution when used as a wash and incubation solution as shown by bar G.

The results of various pH and oxidation-reduction potential induction conditions on excystation are shown in Table I. Optimal results were obtained between pH 1 and 3, above which the excystation fell off precipitously. The corresponding optimal oxidation-reduction potential range was +15 to +135 mV.

The effect of pH adjustment of the trypsin-Tyrode's wash and incubation solution, when all other parameters were held constant, was determined. When this solution was at a pH of 8·0, the excystation results were optimal. At this pH the oxidation-reduction potential was +26 mV. As the solution was adjusted downward from pH 7, excystation declined.

The effect of exposure time in the reducing solutions during induction is depicted in Fig. 2. Three different replicates including all time points run on different days are represented. To examine the relationship between percentage excystation and induction time, a least square regression analysis was run. The graph shows a gradual decline in excystation potential ($\beta = -0{\cdot}2$, $p = 0{\cdot}03$) from the mid 90th to the mid 80th percentile range. Statistical tests (DRAPER & SMITH, 1966) for this linear relationship showed no significant lack of fit ($p = 0{\cdot}58$, $r^2 = 0{\cdot}52$). The negative slope indicates the shorter the induction exposure time the higher the excystation percentage; however, induction times as long as 30 min allowed consistent excystation in the 90th percentiles. A 30 min exposure time for the rest of the experiments was chosen because of the above results and physical logistics of doing the work.

Fig. 3 is a typical experiment showing the effect temperature had on the excystation process. Each co-ordinate represents the temperature for both the induction and incubation steps. Below 20°C and above 45°C excystation was greatly depressed. Optimum excystation occurred around 37°C.

When cysts were exposed to extremes of temperature before being exposed to optimal excystation conditions, they were generally unable to complete the excystation process. For example, cysts which had been frozen in distilled water at −20°C, thawed and then induced did not excyst. Similarly, cysts that were dropped into 100°C distilled water, removed within 5 min, and induced to excyst did not do so. Since excystation was in sharp decline at 45°C (Fig. 3), it was of interest to try to determine the thermal death point, which is defined as the lowest temperature at which a suspension of micro-organisms is killed in 10

Table I—Effect of induction pH and oxidation-reduction potential on *Giardia muris* excystation

pH	Oxidation-Reduction Potential (mV)	Percentage Excystation
1	+135	93
2	+ 75	85
3	+ 15	95
4	− 10	55
5	− 50	12
6	−110	5
7	−200	2
8	−250	1

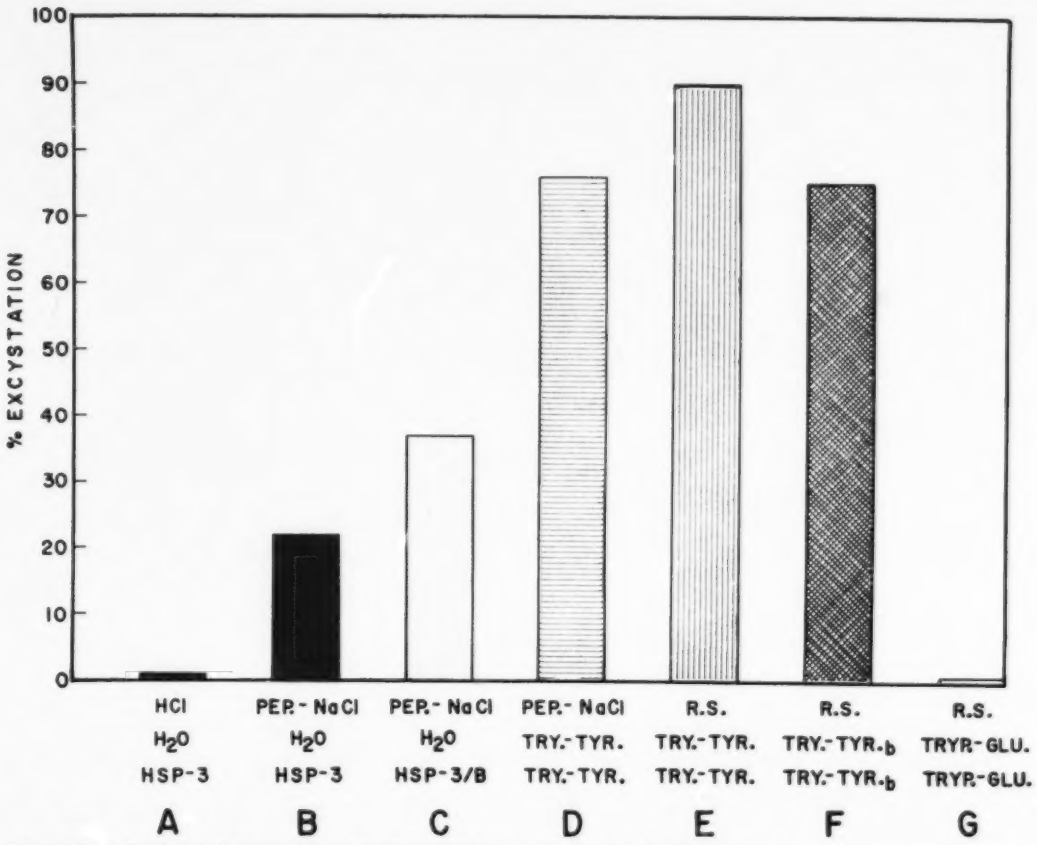

Fig. 1. Effect of various induction, wash, and incubation solutions on *Giardia muris* excystation.
PEP-NaCl = Pepsin Digest Solution, HSP-3/B = HSP-3 Incubation Medium with Mouse Bile, TRY.-TYR. = Trypsin-Tyrode's Solution, TRY.-TYR$_b$ = Boiled Trypsin-Tyrode's Solution, R.S. = Reducing solution (1 part) +0·1 M Sodium Bicarbonate (1 part), TRYP.-GLU. = Trypticase-Glucose Solution.

Table II—Thermal death point determination for *Giardia muris* cysts

Cyst suspension exposed to temperature for 10 min	% Excystation by haemocytometer	# Alive by scanning slide
50°C	0	3
52°C	0	1
54°C	0	0
56°C	0	0
58°C	0	0
22°C (control)	97%	TNTC*

*Too numerous to count

min (CARPENTER, 1967). After pre-exposure in distilled water to the various temperatures indicated in Table II for 10 min, the cysts were subjected to optimal excystation conditions within 5 min. No empty cyst walls were found on counting 100 cysts from any of the temperatures except 22°C, the control. If, however, the entire slide which held approximately 100,000 cysts was scanned for motile trophozoites, some were found at both 50 and 52°C. This indicated that killing was not complete at these temperatures and that our usual counting procedure did not sample a large enough population to distinguish percentages of less than 1. On scanning the slides from the 54, 56 and 58°C in their entirety, no motile trophozoites were found, indicating a thermal death point of about 54°C.

When *G. muris* cysts were extracted from faecal material and were stored in distilled water at 4°C, some were able to remain viable for at least 40 days (see Fig. 4). Cysts freshly isolated from faecal material and induced immediately excysted in the 90 percentile range. Cysts of *G. muris* retained their excystation potential for greater than 85% excystation for nearly two weeks after which their ability declined to less than 40% after 40 days of storage. Phase microscopical observations of three- to six-week-old cyst preparations indicated that cyst walls as well as encysted trophozoites were altered. Refractility changes of the

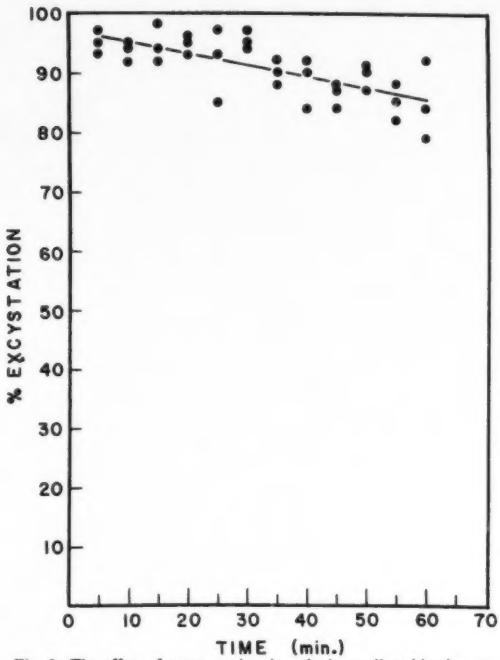

Fig. 2. The effect of exposure time in reducing-sodium bicarbonate solution on *Giardia muris* excystation.

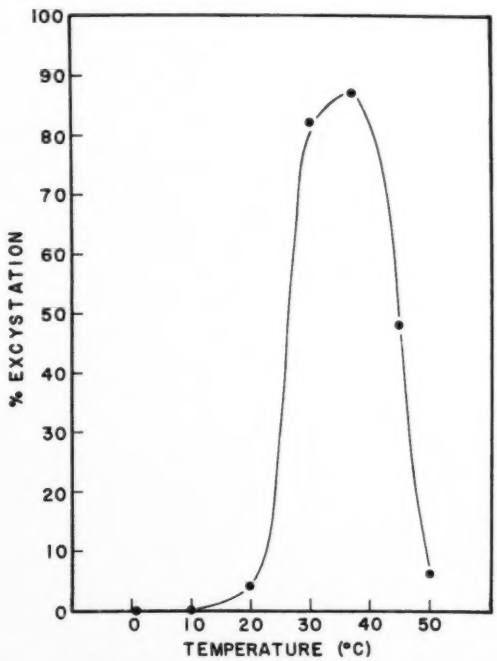

Fig. 3. Effect of induction and incubation temperatures on *Giardia muris* excystation.

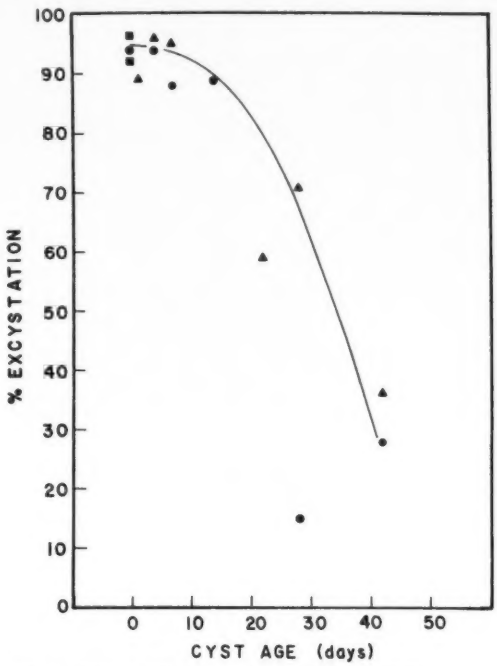

Fig. 4. Effect of cyst age on *Giardia muris* excystation.
■ *G. muris* cysts obtained after 2 hours rather than overnight collection
● Storage experiment #1
▲ Storage experiment #2

encysted trophozoites were very prominent with cysts appearing phase dark rather than the usual phase bright. Cyst walls in many cases showed mycelial-like projections, indicating possible fungal growth. Growth of yeasts, fungi and bacteria in stored preparations may have contributed to decreased viability, for *G. muris* cyst preparations remained viable much longer when microbial growth was less severe.

During optimal *G. muris* excystation, the caudal flagella and distal ends of the other flagella protrude out of the cyst first. Flagellar movement starts slowly, but rapidly increases within 5 to 10 min of emergence. The rapid flagellar movement seems to help in pulling the trophozoite out of the cyst wall. Quadrinucleate trophozoites just after emergence from the cyst wall are rounded and atypical in appearance. Empty cyst walls are easily seen. When seen on end, they give the impressions of a very irregular polar opening that appears somewhat fluted. Cytokinensis begins shortly after the trophozoite has emerged.

Attempts to culture optimally excysted *G. muris* trophozoites in Diamond's TPS-1 medium (VISVESVARA, 1980) have been unsuccessful. Even with the inclusion of standard antibiotics, the cultures were eventually overwhelmed by contaminating yeasts within two to three days.

Discussion

Excystation of *G. muris* cysts is induced by

physiological temperature, the presence of CO_2, low pH and positive oxidation-reduction potentials. Although these conditions are similar to those reported by BINGHAM *et al.* (1979) and RICE & SCHAEFER (1981) for *G. lamblia* excystation, they are not identical. In the case of *G. muris*, inorganic acids are not required, since only the organic constituents of the reducing solution were required in this study. Furthermore, BINGHAM *et al.* (1979) did not report reducing conditions and CO_2 as essentials of *G. lamblia* induction as shown here for *G. muris* (Fig. 1) and as RICE & SCHAEFER (1981) did for *G. lamblia*. Over-all, however, the induction conditions for the two *Giardia* species successfully excysted to date appear to mimic what occurs in the host stomach. The pH of the mammalian stomach ranges between 1·5 and 2·5 (PROSSER & BROWN, 1961). Optimal pH for induction of both *G. lamblia* and *G. muris* is around 2. BERGEIM *et al.* (1945) reported that the oxidation-reduction potential for the rat stomach averages around +150 mV, which approximates the optimal induction condition reported here. Carbon dioxide is found free in the lumen of both the stomach and small intestine. CALLOWAY (1968) reported that the percentage by volume of CO_2 in the stomach can range from 3·9 to 75·2, depending upon the mammalian species. In our procedure the mixing of the bicarbonate and the reducing solutions during induction results in CO_2 evolution. If the tube was not securely capped during induction, the evolved CO_2 escaped and the excystation percentage always decreased. While separate induction temperature studies were not conducted, the combined induction-incubation temperature studies demonstrated that physiological temperature was required for optimal excystation activity. Clearly, there is nothing aberrant about the induction conditions used in this study, compared with normal conditions in the mammalian stomach.

As materials move from the stomach into the small intestine, the conditions encountered change radically with regard to pH and oxidation-reduction potential. MAGEE (1962) reported that the pH of the duodenum, where Brunner glands are located, is usually between 7 and 8 in most species. Following meals, the proximal small intestine becomes more acidic (WORMSLEY, 1971), ranging between 4 and 5. FORDTRAN & LOCKLEAR (1966) stated that there is a pH gradient in the small intestine with the terminal sections being approximately neutral. Gradual neutralization of materials coming from the stomach occurs by the time the terminus of the small intestine is reached. In the present study, trypsin-Tyrode's solution (pH 8) probably was beneficial in neutralizing the acidity of the induction solution and was incorporated into the wash step. METTRICK & PODESTA (1974) have reported that an oxidation-reduction gradient exists in the small intestine as well. The proximal oxidation-reduction potential is around −50mV, while the terminal section is around −200mV. In our work the washing step with trypsin-Tyrode's solution is similar to the neutralization that occurs in the small intestine. While the incubation procedure we used was static, it did provide a near neutral pH and very low electropositive oxidation-reduction potential. Both conditions can be found in portions of the small intestine and mimic *in vitro* what is occurring *in vivo*.

LACKIE (1975) reviewed the methods that had been used to activate infective stages of vertebrate endoparasites *in vitro*. Succinctly, the important chemical and physical factors for protozoans included temperature, pH, reducing conditions, gases, enzymes and bile. Generally, temperatures of about 37°C, like that for *Giardia* spp., were important in all cases except for coccidian forms in birds which have a body temperature between 39 and 41°C. The coccidians from birds have adapted to the higher physiological temperature. Unlike *Giardia* spp., other protozoan forms do not seem to require activation by exposure to low pH. However, they can tolerate exposure to acid conditions, because they normally pass through the stomach during the infective process. Reducing conditions were as important a trigger for the amoebae and coccidians as they are for *Giardia* spp. Digestive enzymes were important for all the protozoans studied to date except *Giardia*. Generally the enzymes required were either trypsin or trypsin plus lipase. Trypsin, although not essential in this study, did seem to accentuate excystation. In no case has pepsin been reported as a requirement. Bile is of paramount importance only for the coccidians.

A requirement for cyst maturation has been reported for *G. lamblia* (BINGHAM *et al.*, 1979) and *G. muris* (see IWANCZUK, 1965) before optimal excystation and infection, respectively, can occur. BINGHAM *et al.* (1979) found that a period varying between 2 and 7 days following faecal isolation was required. After the "maturation period", levels of excystation routinely increased. Similarly, RICE & SHAEFER (1981) found the same to be true and always matured their cysts for one week after isolation. Storage in both laboratories was in suspension at 4°C. IWANCZUK (1965) reported a maximum infectivity response from *G. muris* cysts after they had been stored in mouse faecal material at ambient temperatures between 2 and 24 hours. In the present study we were unable to confirm this requirement for excystation. In our studies, *G. muris* cysts isolated either after collection overnight or for two hours or less excysted at equally high levels. For both faecal collection periods the cysts were isolated as rapidly as possible to minimize exposure to faecal material. Freshly purified *G. muris* cysts have been routinely used for maintaining the mouse infections in this laboratory. No difference in infectivity has ever been noticed between *G. muris* cysts a week or so old and those freshly isolated. Differences between strains of *G. muris* may account for these contradictory findings.

Although *G. muris* excystation can be reliably effected, the process is not completely understood. The unknown chemical composition of the cyst wall continues to be a major stumbling block in this work. It is not known what role unidentified substances in our crude trypsin-Tyrode's solution play in stimulating excystation during the incubation step. Further, we have no idea whether or not our induction procedure triggers some endogenous enzyme(s) associated with the encysted trophozoites. In addition, we have no knowledge of how well or if *in vitro* excystation, which at present is only a measure of viability, indicated infectivity *in vivo*. Work attempting to answer some of these questions continues.

The mechanism of *G. muris* excystation is not completely understood, yet the procedure can be

reliably utilized in its present form as a bioassay system for determining cyst survival.

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